Docket No.12090-03USA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Hyun J. Hwang *et al.*

Application No.: 10/801,342 | Confirmation No.: 2316

Filed: March 15, 2004 | Art Unit: 1637

For: *METHOD AND APPARATUS FOR AMPLIFICATION OF NUCLEIC ACID SEQUENCES BY USING THERMAL CONVECTION* | Examiner: Pande, Suchira

DECLARATION PURSUANT TO 37 CFR 1.131

The undersigned declare as follows:

1. We are co-inventors of the above-identified application (hereinafter «subject application»). In particular, Hyun Jin Hwang is Chairman and Chief Executive Officer of Ahram Biosystems, Inc. of Seoul, Korea; Jeong Hee Kim is Professor at the Kyung Hee University of Seoul, Korea as well as Scientific Advisor of Ahram Biosystems, Inc. of Seoul, Korea; and Kyunghoon Jeong is R&D Manager at Young Lin Instrument Co., Ltd. of Anyang, Korea.

2. The subject application discloses, among other things, an apparatus and method for amplifying nucleic acid sequences. The subject application discloses, for example, an apparatus with a plurality of spatially defined heat sources that supply or remove heat from a sample and create thermal convection conditions for amplifying the nucleic acid sequences.

3. The invention described and claimed in the subject application was conceived and reduced to practice in the Republic of Korea prior to March 9, 2001.

4. For instance, and well before March 9, 2001, at least one of us recognized the need to make a nucleic acid sequence amplification apparatus as described in the subject application that uses a convection-based polymerase chain reaction (PCR) to amplify nucleic acid sequences. More specifically, at least one of us saw that the apparatus should have, among other features, *a plurality of heat sources which supply heat to or remove heat from a plurality of specific regions in a sample*. In support, we attach as **Appendix 1** a true and accurate copy, with dates and irrelevant information deleted, of a Report made by one of us well before March 9, 2001. The Report shows in the upper left side, among other things, a drawing in which a first heat source (heating block labeled F) and a second heat source (cooling block labeled V) are envisioned for supplying heat to or removing heat from a plurality of specific regions in sample tubes.

5. In the Report shown in **Appendix 1**, reference to «non-cycling convection PCR» and «Solution C-PCR» refer to convection PCR as disclosed and claimed throughout the subject application.

6. The Report shown in **Appendix 1** also shows, among other things, conception by at least one of us well before March 9, 2001 of an apparatus for performing convection PCR in which *the plurality of heat sources are arranged such that a first heat source providing heat to a lower portion of the sample is located lower in height than a second heat source removing heat from an upper portion of the sample*. In particular, the Report shows in the upper left side a drawing in which the first heat source (heating block labeled F) is located below the second heat source (cooling block labeled V). The Report also shows, among other things, that at least one of us conceived of particular heat source dimensions before March 9, 2001 (see upper top, left; first heat source height = 25 mm; second heat source height = 20 mm).

7. The Report shown in **Appendix 1** further shows, among other things, recognition by at least one of us well before March 9, 2001 that there was a need to vary sample hole depth in the first and second heat sources (labeled A, B and C with depths 5, 10 and 15 mm, respectively) to identify a heat source configuration that would create *a spatial temperature distribution with spatial regions fulfilling temperature conditions suitable for convection PCR* in the apparatus (see also the bottom of **Appendix 1** and ¶ 20 herein).

8. The Report shown in **Appendix 1** further evidences, among other things, conception by at least one of us well before March 9, 2001 of suitable heat source temperatures and other parameters for performing convection PCR with the apparatus described in the subject application. In particular, at least one of us conceived that the lower (first) heat source could have a heating temperature of 98°C and that the upper (second) cooling source could have a range of suitable temperatures (30°C, 40°C, 50°C, and 60°C). As also shown in the Report, at least one of us identified additional parameters useful for performing convection PCR with the apparatus including varying the depth of the sample hole in the first heat source from 5 mm to 15 mm; and having a polymerization time of 2 hrs, a reaction volume of 50 microliters, an enzyme (Taq) amount of 0.27 pmole, a 65-mer template in an amount of 25 fmol, primer composition (KS, SK) and amounts of 10 pmole each, and a dNTP amount of 1 nmol (see middle of the Report).

9. Well before March 9, 2001, at least one of us (or another acting under our direction and supervision) made a working example of the nucleic acid sequence amplification apparatus for performing convection-based PCR as described in the subject application (hereinafter «apparatus»). Attached as **Appendix 2** is a true and accurate copy of a photograph of the apparatus (disassembled) showing, among other things, the second heat source (left), *an insulator to be positioned between the first and second heat sources* (center, with connecting bolts), and the first heat source with sample hole depths of 5 mm, 10 mm and 15 mm (right).

10. The **Appendix 2** also shows, among other things, that well before March 9, 2001 at least one of us recognized the need to include a copper inlet tube and a copper outlet tube in the second heat source (left). These components allowed *a liquid* such as water to be *in thermal contact with a specific region of the sample* in contact with the second heat source. As further shown in Appendix 3 (below), the second heat source was further made before March 9, 2001 to include a *receptor* (embedded between the sample tube holes inside the second heat source) *in which the liquid was contained to remove heat from the sample*.

11. **Appendix 3** is a true and accurate copy with dates and irrelevant information removed of a schematic drawing made by at least one of us (or by another under our direction and

supervision) well before March 9, 2001 that shows, among other things, a horizontal view of the second heat source of the apparatus. The drawing shows, among other things, the receptor (shown with dotted lines) for containing the liquid, embedded between the sample tube holes within the second heat source of the apparatus. In the drawing, «A» indicates a drilled hole that was sealed by soldering with a copper rod disk after the drilling and «B» indicates a drilled hole that was soldered to a copper tubing after the drilling to provide the copper inlet and outlet tubes. The inlet and outlet tubes and receptor of the second heat source shown in **Appendices 2 and 3,** for example, were designed by at least one of us to produce or assist *a circulation unit that would circulate liquid in the apparatus*.

12. The **Appendix 2** also illustrates, among other things, that well before March 9, 2001 the apparatus included *a plurality of heat sources with a first thermally conductive solid in thermal contact with a lower portion of the sample and a second thermally conductive solid in thermal contact with an upper portion of the sample*. In the apparatus shown in **Appendix 2,** the first and second thermally conductive solids include copper to facilitate heat transfer.

13. **Appendices 1** and **2** further show, among other things, that well before March 9, 2001 at least one of us conceived and made a nucleic amplification apparatus in which *at least one of the heat sources comprises a thermally conductive solid in thermal contact with a specific region of the sample*. See the upper left part of Appendix 1 (particularly the two heat source blocks labeled F and V which include sample holes labeled A, B, and C): and Appendix 2.

14. The **Appendix 2** also shows, among other things, that well before March 9, 2001, the apparatus we made included *an opening defined by the first and second heat sources and the insulator*. The opening is adapted to receive a reaction vessel with sample. Referring again to **Appendix 2**, the apparatus has nine (9) of such openings that are defined by the second heat source (left), the insulator (middle) and the first heat source (right). Three (3) reaction vessels received by the openings in the apparatus are shown (right).

15. The **Appendix 2** also shows, among other things, that well before March 9, 2001, the opening for receiving the reaction vessels included *a first through hole within the second heat*

source (left), *a second through hole within the insulator* (middle) and an opening within the first heat source (right). *The opening is essentially perpendicular to the insulator* and *it is adapted to receive a reaction vessel configured as a straight cylinder or tube* (3 such tubes are shown, right).

16. **Appendix 4** is a true and accurate copy with dates and irrelevant information removed of a schematic drawing made by at least one of us (or by another under our direction and supervision) well before March 9, 2001 that shows, among other things, a vertical section taken through certain sample tube holes of the first heat source to show *a closed bottom end within the first heat source*. The plane of section was taken through sample tube holes of equal depth within the apparatus.

17. **Appendix 5** shows, among other things, the apparatus made by at least one of us (or by another under our guidance and supervision) well before March 9, 2001 as an assembled unit.

18. Well before March 9, 2001, at least one of us prepared a Report with reaction conditions to test the apparatus we had made under convection PCR conditions. **Appendix 6** is a true and accurate copy, with dates and irrelevant information removed, that shows, among other things, reaction conditions designed by at least one of us for confirming that the apparatus could amplify nucleic acid under convection PCR conditions. In particular, the Report shows, for instance, data from an experiment in which reaction tube type (glass) and depth (8mm), template type and amount, buffer and magnesium chloride concentration, primer pair type, dNTP amount, polymerase enzyme type, reaction volume (100 µl) and reaction time (0.5, 1, 2, and 4 hrs) are specified.

19. In the Report shown in the **Appendix 6**, reference to «C-PCR-soln» at the top means convection PCR in solution as described in the subject application.

20. The **Appendix 6** also shows, among other things, that well before March 9, 2001 at least one of us used the apparatus to perform convection PCR and amplify the pbs template. In particular, the lower left portion of **Appendix 6** shows a schematic representation of the sample

holes of the second heat source (see Appendix 2) in which numbers 1, 2, 3, and 4 mark sample tube holes subjected to reaction times of 0.5, 1, 2, and 4 hours, respectively. **Appendix 6** further shows, in the lower right portion, a photograph of a stained agarose gel showing amplification of the pbs template between 1 and 6 hours (lanes 2, 3, 4, and 5 from the left side of the gel). The band present in the control lane (0 hours) is a dimer band artifact unrelated to the specific amplification signal we found (just above the dimer band) between 1 and 6 hours.

21. At least one of us concluded from the results shown in **Appendix 6**, for instance, that the apparatus we made and tested well before March 9, 2001 successfully amplified the pbs template by convection PCR. At least one of us further concluded from this and related experiments performed by at least one of us that for a 100 microliter reaction volume a sample hole depth of 8 mm (exluding the wall thickness of the tube on the bottom) within the first heat source was sufficient to support convection PCR with the apparatus.

22. Well before March 9, 2001, at least one of us detected a spatial temperature distribution within the apparatus (in use). More specifically, at least one of us quantified the spatial temperature distribution within: (1) the sample hole having a depth of about 30 mm and (2) the sample tube of glass at a depth of 8 mm in the first heat source (Appendix 6). **Appendix 7** is a true and accurate copy with dates and irrelevant information deleted showing, among other things, a Report in which at least one of us determined the spatial temperature distribution inside the sample holes of the first and second heat sources (left hand column of temperature measurements). The Report also includes a drawing (middle) showing a representation of the sample tube divided into millimeter increments from the bottom of the sample tube in the first heat source (0 mm) to the top in the second heat source (about 30 mm). The Report also shows, among other things, data from the same experiment in which the spatial temperature distribution inside the sample itself (right hand column of temperature measurements) was quantified.

23. In particular, the Report shown in **Appendix 7** shows, among other things, that in this experiment, the first heat source of the apparatus had an operational temperature of 92.6°C and the second heat source («top block») had an operational temperature of 50°C (see information below the right hand column of temperature measurements).

24. At least one of us concluded from the data presented in **Appendix 7**, for instance, that the apparatus we made and tested well before March 9, 2001, could produce *a spatial temperature distribution with spatial regions fulfilling temperature conditions suitable for i) a denaturation step in which double strand DNAs become separated to single strand DNAs .* Referring again to **Appendix 7**, at least one of us concluded that the denaturation step could occur, for instance, between 0 mm to about 5 mm in the sample (temperature between 93.9°C to 87.4°C) under the reaction conditions used.

25. At least one of us further concluded from the data presented in **Appendix 7,** for instance, that the apparatus we made and tested well before March 9, 2001 could produce *a spatial temperature distribution with spatial regions fulfilling temperature conditions suitable for (ii) an annealing step in which the single strand DNAs formed in the denaturation step hybridize to the primers to form DNA-primer complexes.* Referring again to **Appendix 7**, the annealing step could occur, for instance, between about 20 to about 30 mm in the sample (temperature between about 53.6°C to 50°C).

26. At least one of us further concluded from the data presented in **Appendix 7**, for example, that the apparatus we made and used well before March 9, 2001 could produce *a spatial temperature distribution with spatial regions fulfilling temperature conditions suitable for (iii) a polymerization step in which the primers in the DNA-primer complexes are extended by the polymerization reaction.* Referring again to **Appendix 7**, at least one of us concluded that the polymerization step could occur, for example, between about 10 mm to about 15 mm in the sample (temperature between about 72.6°C and 60.9°C in the sample) or between about 5 mm to about 15 mm in the sample (temperature between about 87.4°C and 60.9°C in the sample).

27. At least one of us further concluded from the data presented in **Appendix 7,** for instance, that the apparatus described in the subject application and which we made and used well before March 9, 2001 could produce *a spatial temperature distribution that induces circulation of the sample by thermal convection so that the denaturation, annealing and polymerization steps occurred sequentially and repeatedly inside the sample.* Referring again to **Appendix 7**, the

spatial temperature distribution found in the sample tube could induce circulation in the sample, thereby creating the thermal convection needed to denature, anneal and polymerize multiple times to produce the amplification we observed (see Appendix 6 data).

28. At least one of us further concluded from the data presented in **Appendix 7**, for instance, that *the heat sources* of the apparatus we made and used well before 9 March 2001, *were arranged to provide for a spatial temperature distribution that included a convection region positioned between a relatively high temperature region and a relatively low temperature region*. Referring to the data presented in **Appendix 7,** at least one of us concluded from the spatial temperature distribution data that the convection region was position between from 0 mm to about 5 mm (high temperature region) and from about 20 mm to about 30 mm (low temperature region) within the sample tube.

29. Well before March 9, 2001, at least one of us conceived of a reaction vessel configured to fit within the apparatus we made and tested well before that date. For instance, *a reaction vessel with a single passage between the relatively high temperature region and the relatively low temperature region* was used in our apparatus. The **Appendix 1** shows, among other things, the Report made well before March 9, 2001 showing an example of such a reaction vessel having an outer diameter (Φ) of 7.9 mm, and an inner diameter (Φ) of 2 mm with vessel wall thickness of about 3 mm (top right).

30. In particular, and well before March 9, 2001, at least one of us concluded from the data presented in the **Appendices 1, 6 and 7**, for example, that the single passage of the reaction vessel is adapted to contain the convective flow (upward and downward) that we detected and identified within our apparatus when used. We further concluded from this information that a reaction vessel with a single passage used in our apparatus achieved nucleic acid amplification through bidirectional convection (i.e., both upward and downward convection) within the reaction vessel as explained throughout the subject application.

31. The **Appendices 1, 2, and 5,** for instance, further show, among other things, a reaction vessel positioned vertically with respect to the heat sources. Also shown is a reaction vessel suitable for use with the apparatus with a top and bottom end in which the bottom end is closed.

32. The **Appendices 1, 2, and 5,** further show, among other things, that the apparatus made and tested well before March 9, 2001 could accommodate multiple reaction vessels.

33. **Appendix 1** further shows that well before March 9, 2001 at least one of us conceived of the need to introduce a vertical gap between the top of the relatively high temperature region and the bottom of the relatively low temperature region. See the vertical gap (air) of about 3 mm between heat sources V and F of Appendix 1 (top left). The vertical gap is shown as a solid in **Appendix 2** (middle). **Appendix 1** also provides for a gap between the sample and at least the second heat source. See top middle showing about 3 mm gap between the sample and the edge of the sample tube (next to heat source).

34. We hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: SEPT. 26, 2008 Name: Hyun Jin Hwang

Hyun Jin Hwang

Date: Sept. 26. 2008 Name: Jeong Hee Kim

Jeong Hee Kim

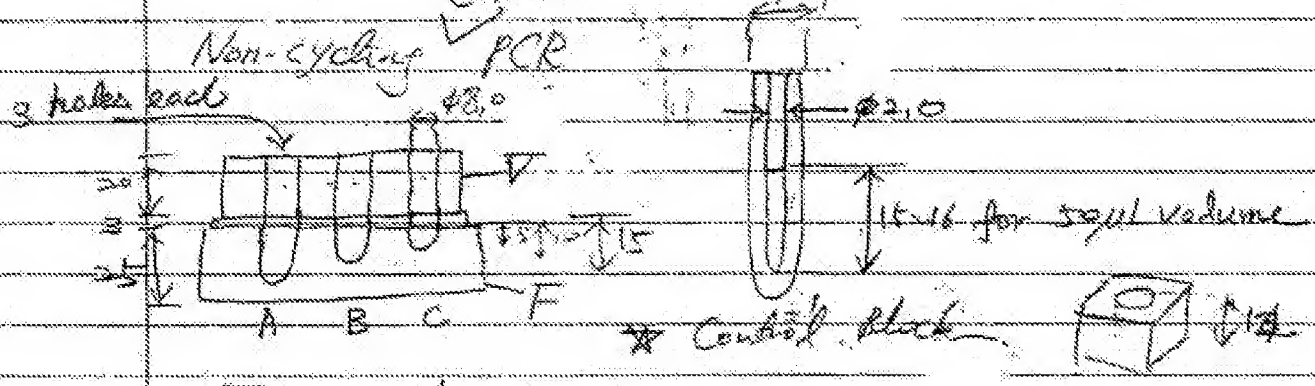
Date: Sept. 27. 2008 Name:

Kyunghoon Jeong

★ (Next report will be finish Soln-C-PCR experiments and prepare for BIM-C-PCR.

Convection

Non-cycling PCR



Try condition:

F (control) = 98°C ; A, B, C have different F temperature

V = 30, 40, 50, 60°C

Polymerization time : 2 hrs

★ Solution C-PCR conditions

- Total volume : 50 μL
- Taq : 0.25 μL each = 0.37 pmol
- template : 5 mer, as final
- primer : K5 & 5K, 10 pmol each
- dNTP : 1 mmol

★ Control : Solution PCR (Gel-Standard loading)

★ DNA Marker : check → densitometry of band

★ Gel prep. : Technique

Gel %m, o/o, loading, vol.-current time, expose time, dye composition, concentration

★ Densitometry

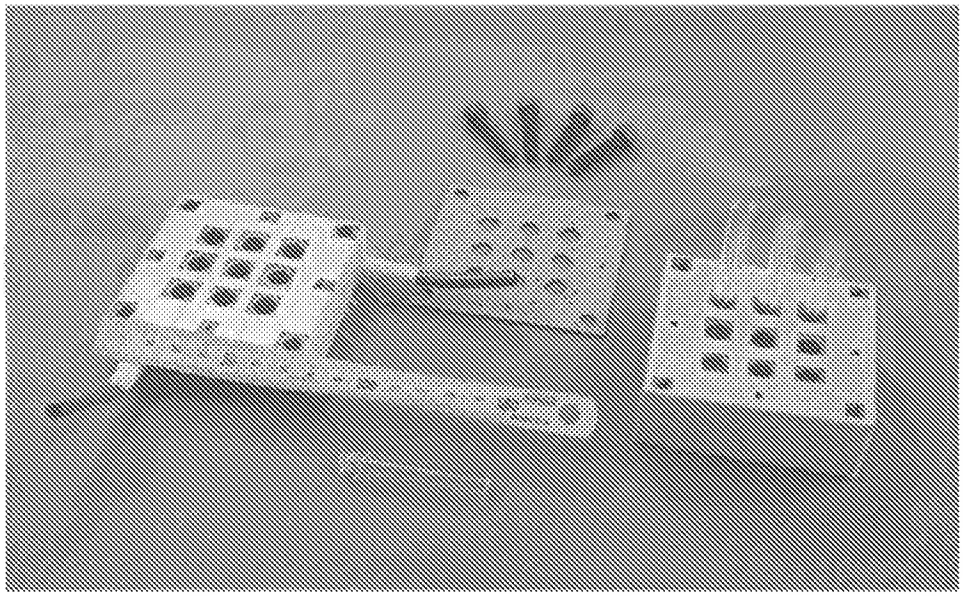
2nd try : (1) select appropriate depth (F).

(2) Vary F temp systematically.

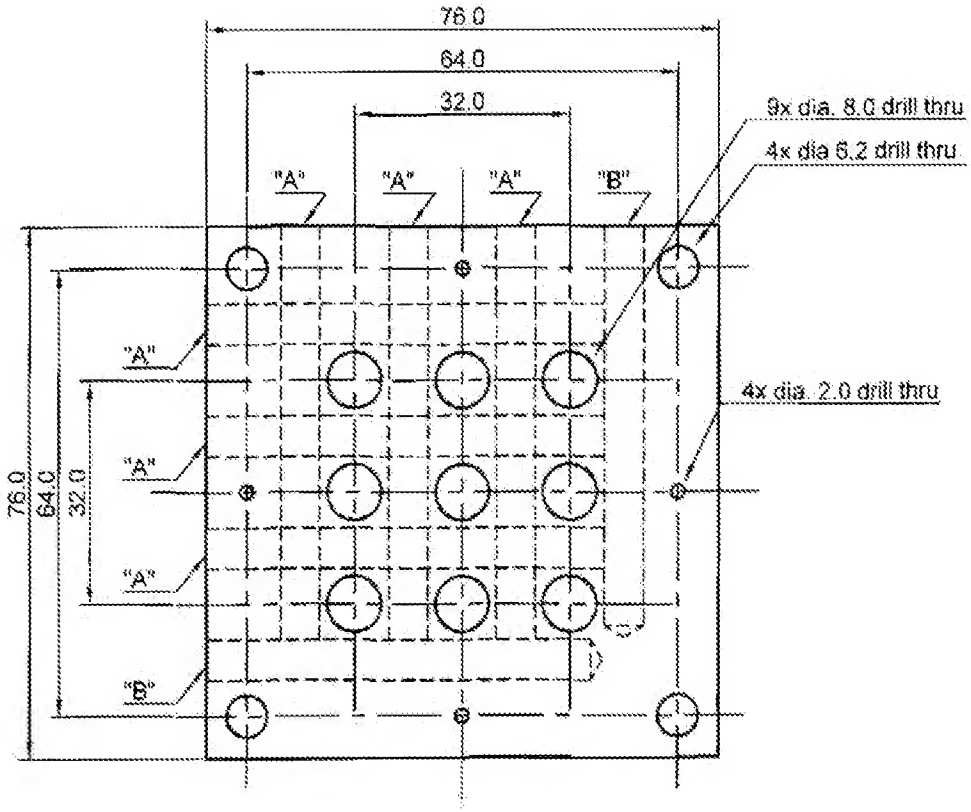
For each F temp, vary V Temp. Systematically

3rd try : Depth scan after knowing F & V temperature conditions

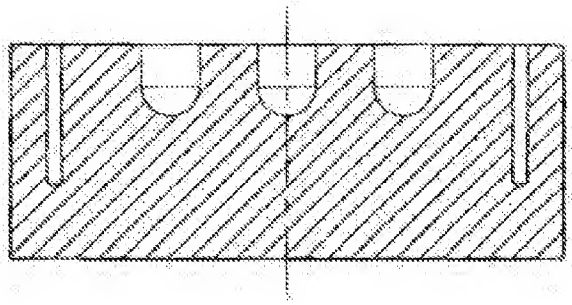
Appendix 1



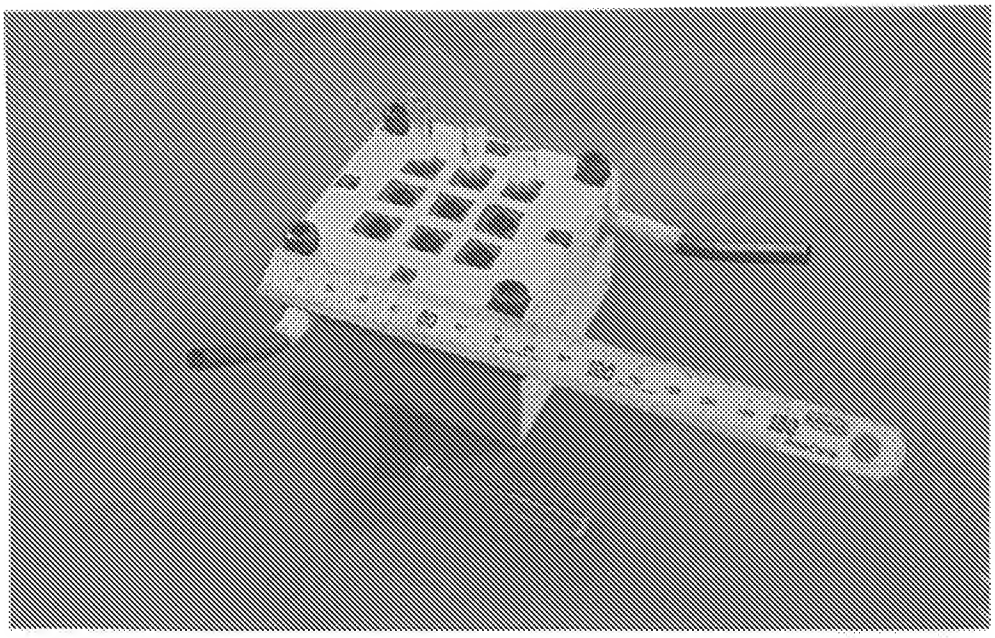
Appendix 2


76.0
64.0
32.0
9x dia. 8.0 drill thru
4x dia 6.2 drill thru
"A"
"A"
"A"
"B"
"A"
"A"
"A"
"B"
4x dia. 2.0 drill thru
76.0
64.0
32.0

Appendix 3



Appendix 4



Appendix 5

NC.

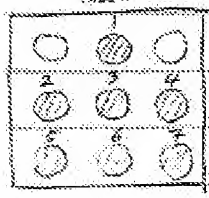
* C-PCR -soln. Time dependance

glass tube

Depth 8 mm. Rxn. 100μl

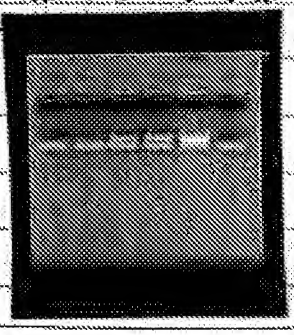
Time. 0.5 hr. 1 hr 2 hr 4 hr

	ea.	× 42	× 33 modified
Set up			
Template pBS 1ng/μl	1	42	33 ✓
10x buffer	10	42	33 ✓
10x $MgCl_2$	10	42	33 ✓
SK primer 10μM	2	8.4	6.6 ✓
KS primer 10μM	2	8.4	6.6 ✓
dNTP 2.5mM	8	33.6	26.4 ✓
Taq pol	2	8.4	6.6 ✓
dH_2O	65	273	214.5 ✓
	100μl		



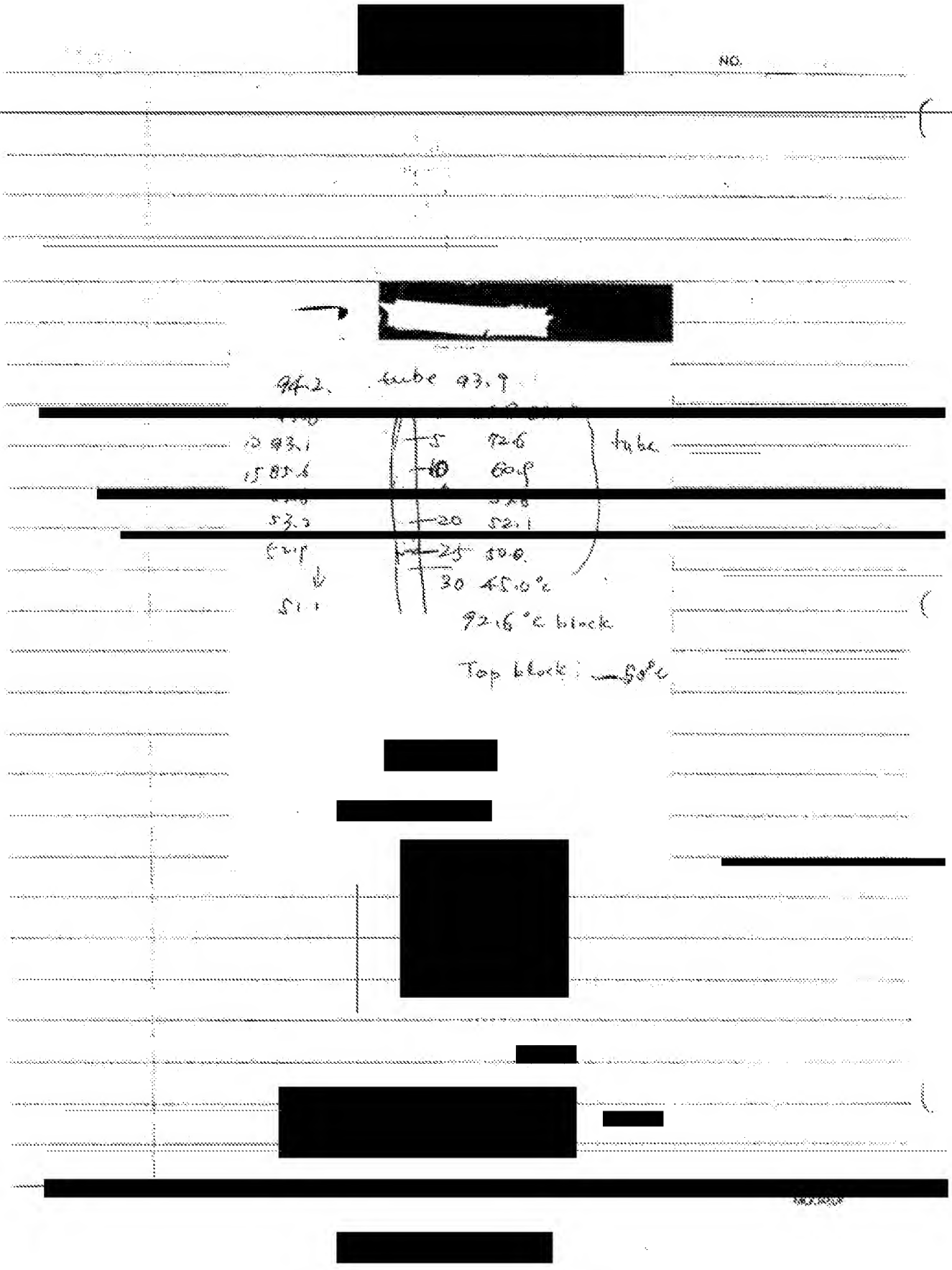
tube 1 0.5 hr
2 1 hr
3 2 hr
4 4 hr

start 2:35 pm



soln line

Appendix 6


NO.
tube 93.9
5 92.6
10 60.9
20 52.1
25 50.0
30 45.0°c
92.6°c block
Top block: 50°c

Appendix 7